

1 **In- vitro-activity of additive application of hydrogen peroxide in antimicrobial**
2 **photodynamic therapy using LED in the blue spectrum against bacteria and biofilm**
3 **being associated with periodontal disease**

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Abstract

Background: Although antimicrobial photodynamic therapy (aPDT) has been shown to be efficient in killing planktonic periodontopathogenic bacteria, its activity on established biofilms is very limited. The aim of the present in-vitro study was to evaluate the potential effect of hydrogen peroxide as a pretreatment for aPDT.

Methods: aPDT consisting of riboflavin as photosensitizer and illumination by a LED lamp emitting in the blue spectrum for 30 s and 60 s (aPDT60) was combined with a pretreatment with 0.25% and 3% hydrogen peroxide. The antimicrobial activity of these treatments was determined against eight oral species (incl. *Porphyromonas gingivalis* and *Tannerella forsythia*) and against eight-species biofilms. Treatment of biofilms in an artificial pocket model included a mechanical removal of the biofilm.

Results: Against planktonic bacteria, pretreatment with hydrogen peroxide increased killing of planktonic bacteria, after aPDT60 no viable bacteria were detected in 7 of 8 strains. In biofilms formed on well-plates, aPDT60 reduced bacterial counts only by 0.53 log₁₀ cfu, whereas reduction was closed to 4 log₁₀ or higher when 3% hydrogen peroxide was used. When biofilms were treated in the periodontal-pocket model, reduction of cfu was less than 0.5 log₁₀ after mechanical therapy or aPDT60 only, however no bacteria were detected after mechanical biofilm removal followed by the use 3% of hydrogen peroxide and aPDT60.

Conclusions: aPDT using riboflavin and blue LED light applied after mechanical removal of biofilm and adjunctive 3% hydrogen peroxide solution appears to represent an alternative for antimicrobial periodontal therapy.

Key words: biofilms; riboflavin; antimicrobial photodynamic therapy; hydrogen peroxide; periodontopathogens

1. Introduction

The pathogenesis of periodontitis is thought to be an inflammatory response to the microbiota in subgingival biofilm [1]. It has been repeatedly shown that the mechanical removal of bacterial biofilm is the most essential part of periodontal therapy [2]. In addition, a number of various antimicrobials such as chlorhexidine digluconate (CHX) are widely used as adjunctive to mechanical debridement [3, 4]. Furthermore, several systematic reviews have provided evidence for the efficacy of amoxicillin and metronidazole as adjuncts to scaling and root planing in nonsurgical therapy of chronic periodontitis [5, 6]. However, in the light of the dramatic global increase in antibiotic resistance the unnecessary use of antibiotics should be avoided [7].

Light-activated killing appears to be an interesting approach to overcome the problems related to the use of antibiotics [8]. In antimicrobial photodynamic therapy (aPDT), singlet oxygen and other reactive oxygen species being highly toxic to microorganisms are generated through the activation of photosensitizers by light [9]. Light sources in aPDT are diode lasers [9] or light-emitting diodes (LED) [10, 11].

In the last few years, the use of LED in conjunction with aPDT became more and more popular. Several in-vitro studies have provided evidence for an antimicrobial activity when combined with the respective photosensitizer, *e.g.*, LED emitting in the red spectrum with methylene blue [12] or with toluidine blue [13]. LED emitting in the blue light are routinely used for photopolymerization in dentistry [14]. When combined with curcumin as photosensitizer LED emitting in the blue spectrum kills efficiently bacteria [15]. In recently published in vitro studies [11, 16] we determined the effect of aPDT using LED in the red and in the blue spectrum on planktonic microorganisms and being in a biofilm, which are involved in the pathogenesis of periodontitis. The findings indicated that aPDT using LED in the red spectrum was active against periodontopathogenic microbial species [11]. In a second study LED emitting in the blue light was tested. The used device was designed both for curing composite materials based on camphorquinone as photoinitiator as well as for photodisinfection. Another advantage was due to the colorless natural photosensitizer (riboflavin). aPDT using LED

emitting in the blue spectrum combined with riboflavin was also active against planktonic periodontopathogens, however, it was inferior to aPDT using LED emitting in the red spectrum combined with toluidine blue [16]. In both studies it became clear, that multi-species biofilms were not sensitive to aPDT using LED. This raised the question, if a modification of treatment protocol may increase anti-biofilm activity. Recently, pre-use of hydrogen peroxide was shown to increase antibacterial activity of aPDT, when combined with methylene blue [17] or toluidine blue [18] as photosensitizers.

Thus, the aim of the present follow-up study was to evaluate, if mechanical therapy followed by a pretreatment with hydrogen peroxide before applying riboflavin as photosensitizer and LED light in the blue spectrum, is able to eliminate efficiently multi-species biofilm. In a first step, the quantitative killing activity of aPDT with pre-applied hydrogen peroxide on different microorganisms was determined. Subsequently, the effect of aPDT used after rinsing with hydrogen peroxide and mechanical biofilm removal was evaluated on bacterial species within the biofilm formed on polystyrene surfaces and on bacterial species within a “periodontitis” biofilm using our “pocket model” [19].

2. Methods

2.1. Light source and chemicals

The used device was a LED lamp emitting in the blue spectrum with a power peak at 460 nm +/- 10 nm (effect approx. 1W, so 2 W/cm² in intensity; FotoSan460; CMS Dental ApS, Copenhagen, Denmark). To activate photosensitizer times of exposure were 30 s and 60 s, the tips of the LED lamps were in direct contact with photosensitizer during activation.

The used photosensitizer (PS) was the respective one consisting of 0.1% riboflavin (Fotosan blue agent; CMS Dental ApS). Hydrogen peroxide obtained from the pharmacy of the University hospital Bern was used in concentrations of 0.25% and 3%.

2.2. Microorganisms

The following bacterial strains were included in the experiments: *Porphyromonas gingivalis* ATCC 33277, *Tannerella forsythia* ATCC 43037, *Fusobacterium nucleatum* ATCC 25586, *Campylobacter rectus* ATCC 33238, *Prevotella intermedia* ATCC 25611, *Streptococcus gordonii* ATCC 10558, *Actinomyces naeslundii* ATCC 12104 and *Parvimonas micra* ATCC 33270. Strains were maintained on Tryptic soy agar plates (Oxoid, Basingstoke, GB) with 5% of sheep blood. One day before the experiments strains were passaged on a new agar plate. Immediately, before starting experiments, a bacterial suspension according McFarland 0.5 (about 1.5×10^8 microorganisms) was prepared. In case of mixture (biofilm experiments) one part of *S. gordonii* suspension was mixed with two parts of *A. naeslundii* suspension and each four parts of the other bacterial suspensions. Bacterial cultures were always incubated anaerobically at 37°C, only *S. gordonii* was cultured with 5% CO₂.

2.3. Quantitative killing activity of aPDT with pre-exposure to H₂O₂ on microorganisms

One ml of a defined inoculum of microorganisms (10^8 /ml NaCl 0.9% w/v each) was given into 1.5 ml dark tubes. After short centrifuging (7000 g, 2 min), the supernatants were removed. Twenty-five µl of hydrogen peroxide solution or 0.9% w/v NaCl were applied first. Then 25 µl of PS (control 0.9% w/v NaCl) were applied for 1 min.

Then the sample was exposed to light for 30 s or 60 s respectively. The numbers of colony forming units (cfu) were determined after addition of NaCl 0.9% solution.

2.4. Effect of aPDT with pre-exposure to H₂O₂ on a multi-species biofilm

Formation of biofilms in well-plates followed most our recently described protocols [16, 20]. Ninety-six-well-plates were covered with a 1.5% bovine serum albumin (SERVA Electrophoresis GmbH, Heidelberg, Germany) / 0.27% pig gastric mucin (Sigma-Aldrich,

Buchs, Switzerland) mixture for 1 h. The multi-species suspension was mixed with nutrient media (Wilkins Chalgren broth (Oxoid, Basingstoke, GB) containing 5 µg/ml NAD and 5 µg/ml thiamine pyrophosphate (Sigma-Aldrich Chemie GmbH) in a ratio 1 : 9. Thereafter, 200 µl of that was added per well. Biofilms were incubated anaerobically for 5 d. Nutrient broth was exchanged and *P. gingivalis*, *T. forsythia*, *C. rectus* and *P. intermedia* were added again after three days. Then, nutrient media was carefully removed and treatment was applied as before. Immediately thereafter, biofilms were removed by scraping, mixing and suspending in 0.9% w/v NaCl. Then the number of the total viable bacteria was counted by determination of cfu.

Second, quantification of the biofilms was made according to protocols published recently [21]. After washing biofilms, adherent cells were fixed at 60°C for at least 60 min. Thereafter, biofilms were stained with 0.06% (w/v) crystal violet and staining was quantified by using a plate reader.

Third, biofilm metabolic viability was assessed with using alamar blue as a redox indicator [22]. Alamar blue was added in a ratio 1 : 20. After incubation for 1 h at 37°C, absorbance at 570 and 600 nm was measured by using a microplate reader.

2.5. Effect of aPDT with pre-exposure to H₂O₂ after mechanical biofilm removal on a multi-species biofilm in an artificial pocket

Our recently described “pocket model” [19, 23] was used. Dentin specimens were prepared from porcine teeth obtained from a slaughterhouse. The crowns of the teeth were removed and dentin slices of the buccal side of the roots were cut with diamond disks (~ 6 × 12 mm) with a thickness of ~3 mm. The surface properties of the buccal side of the dentin specimens were standardized by grinding with silicon carbide papers of #2400 grit size, corresponding to an abrasive particle size of 6.5 µm. Finally the dentin slices were fixed on plastic disks as described recently [19].

The dentin specimens were covered with the serum albumin / mucin solution as before and thereafter placed in tubes. Suspensions of eight bacterial strains were prepared as

described above, then mixed with nutrient broth in a ratio 1 : 9 and transferred to the tubes. After incubation in anaerobic conditions for 72 h, two thirds of the medium were exchanged and *P. gingivalis*, *T. forsythia*, *C. rectus* and *P. intermedia* were added again. After a final incubation of 48 h, the plastic disks with the dentin specimens were removed from the tubes and transferred to the pocket model where treatment methods were applied.

Test methods were mechanical therapy combined with 0.25% H₂O₂ + PS + 60 s light as well as with 3% H₂O₂ + PS + 60 s light. Controls included mechanical therapy alone, 3% H₂O₂ + PS + 60 s light, PS + 60 s light as well as a negative control with 0.9% w/v NaCl only. In case of mechanical therapy, the instrumentation was made with Gracey curettes (CUR) made of stainless steel (Deppeler SA, Rolle, Switzerland). Specimens were instrumented from apically to coronally by means of 20 strokes using both sites of the curettes. Each side of the curette was used for 12 specimens and then replaced by a new curette. Before instrumentation, after every 10th stroke and at the end of instrumentation, the dentin surfaces in the pockets were rinsed with 2.5 ml of 0.9% w/v NaCl. Thereafter, biofilm samples were taken with a cotton swab, suspended in 0.9% w/v NaCl and numbers of cfu were determined. The different experiments are summarized in Table 1.

2.6. Statistical analysis

All experiments were made in four independent series. To avoid bias by the time samples being exposed to aerobic conditions sequence of applied treatment changed in each series.

Results were compared by using ANOVA with post-hoc Bonferroni by using the software SPSS 25.0 (IBM, Chicago, IL, USA) was used. A *p*-value of 0.05 was considered to be statistically significant. In antimicrobial assays the cfu log₁₀ reductions are of importance, following the focus was presentation of log₁₀ cfu values.

3. Results

3.1. Killing of planktonic bacteria

Using aPDT only, results showed a limited activity when light exposure was 30 s. Even, when applying light for 60 s, counts of *S. gordonii* and *A. naeslundii* were not reduced by 3 log₁₀. When pre-exposing gram-positive bacteria (*S. gordonii*, *A. naeslundii*, *P. micra*) to 3% hydrogen peroxide before applying aPDT, those were totally killed or at least sufficiently reduced. Concentration of hydrogen peroxide seemed to be of importance.

Gram-negative bacteria were more sensitive to aPDT than gram-positive bacteria. Applying light for 60 s reduced bacterial counts at least by 4 log₁₀. Pretreatment with hydrogen peroxide independent of the used concentration increased bacterial killing, when thereafter the PS was illuminated for 60 s, no viable bacteria were detected anymore. The results are presented in Fig. 1.

3.2. Multi-species biofilm

In the untreated biofilms 7.86 ± 0.16 log₁₀ cfu were counted in mean. Log₁₀ counts differed statistically significantly between the treatments (ANOVA, $p < 0.001$). aPDT with 30 s and 60s of light exposure reduced bacterial counts only by 0.39 log₁₀ cfu and 0.53 log₁₀ cfu, which was not statistically significantly different from the untreated control. All other treatments resulted in statistically significant differences from the control (each $p < 0.001$). In the biofilms with combined application of hydrogen peroxide and aPDT, both dependencies on the used hydrogen peroxide concentration and the time of light exposure became obvious. The concentration of hydrogen peroxide seemed to be of more importance, application of 0.25% hydrogen peroxide and aPDT reduced bacterial counts by less than 3 log₁₀, whereas reduction was closed to 4 log₁₀ or higher when 3% hydrogen peroxide were used as pretreatment. Differences between the groups applying 0.25% hydrogen peroxide and 3% hydrogen peroxide were statistically significant (0.25% hydrogen peroxide and aPDT with 60 s of illumination vs. 3% hydrogen peroxide and aPDT with 30 s of illumination: $p = 0.004$; all other $p < 0.001$).

Metabolic activity of biofilms and biofilm quantity were the highest in the untreated controls each. But there was not any statistically significant difference between and to the treatments. Results of the biofilms are shown in Fig. 2.

3.3. *Multi-species biofilm in an artificial pocket model*

Bacterial counts were different after applying the treatments (ANOVA, $p < 0.001$). Instrumentation reduced bacterial counts only by 60% (0.4 log₁₀). No bacteria were detected after mechanical biofilm removal combined the use 3% of hydrogen peroxide before aPDT with 60 s illumination ($p < 0.001$ compared to control). Without instrumentation, applying 3% of hydrogen peroxide before aPDT with 60 s illumination reduced the bacterial counts by 5 log₁₀ ($p < 0.001$), but when using 0.25% hydrogen peroxide and aPDT with 60 s illumination, bacterial counts decreased only by 1.67 log₁₀ cfu. Results are presented in Fig. 3.

4. Discussion

In the present in vitro study the influence of a pretreatment with hydrogen peroxide in two concentrations on aPDT using 0.1% riboflavin as photosensitizer and generating light with a LED lamp emitting in the blue spectrum with a power peak at 460 nm was analyzed. Both the applied power of the LED lamp and the concentration of hydrogen peroxide influenced bactericidal and antibiofilm activities.

The used aPDT consisted of riboflavin as a photosensitizer and a light emitting in the blue spectrum. Blue light itself has been reported to exert some antimicrobial activity, but riboflavin increases additionally the bactericidal activity [24]. Riboflavin is an essential nutrient (vitamin B₂) for humans; it functions as an antioxidant against oxidative stress [25]. Comparing different lights, blue light is highest efficient in the production of superoxide radicals from riboflavin which leads to DNA damage of *Escherichia coli* [26]. Charged flavin derivatives which show a better attachment to bacterial cell surfaces compared with riboflavin interact with cell wall components after illumination as shown in transmission electron microscopy photographs

1 of *Staphylococcus aureus* [27]. The results of our study using aPDT with riboflavin on
2 planktonic bacteria confirmed our earlier results about a dependency of the power (time of
3 illumination) and a higher sensitivity of gram-negative bacteria than of gram-positive ones [16].
4 This suggests a particular affinity to the outer membrane of gram-negative bacteria. In literature
5 only data about a photodegradation product of riboflavin as photosensitizer are available, here
6 in contrast activity on a gram-positive bacterium was ten-fold higher than on a gram-negative
7 one [28].

8 Several attempts are made to increase the bactericidal activity of aPDT. One possibility
9 is the development of new photosensitizers, while another is the combination with other
10 compounds. Applying silver nanoparticles together with riboflavin as photosensitizer increased
11 the bactericidal activity of aPDT on *Streptococcus mutans* but not on *E. coli* [29]. Antimicrobial
12 peptides were used together with rose bengal as photosensitizer and the followed illumination
13 killed efficiently a multi-resistant *Pseudomonas aeruginosa* strain [30].

14 In the present study, hydrogen peroxide augmented the activity of aPDT. Hydrogen
15 peroxide is known as a strong oxidant reacting with selected thiol-proteins and transition
16 metals, it may cause biological damage by production of free radicals [31]. Flavins act as
17 benign catalysts of oxidation of selected molecules with hydrogen peroxide [32]. When
18 applying 3% hydrogen peroxide before illumination 60 s, seven of the eight tested bacteria
19 were killed. Our result suggests that the activity of aPDT depended on the concentration of
20 hydrogen peroxide in case of grampositive bacteria, whereas illumination time influenced more
21 aPDT on gram-negative one. Most resistant was *S. gordonii* known as a commensal. The
22 species being catalase negative produces itself hydrogen peroxide which is the alpha-
23 hemolysin [33]. Interestingly, also hydrogen peroxide might act as a photosensitizer. Although
24 there was nearly no bactericidal activity of 0.3 mM hydrogen peroxide on *P. gingivalis* and *F.*
25 *nucleatum* illumination with blue light killed all *P. gingivalis* and reduced *F. nucleatum* counts
26 by about 2 log₁₀ cfu [34]. In our study the solely activity of hydrogen peroxide without and
27 combined with light only was not tested which might be a limitation and an interesting approach

1 for further research. In an experimental series each all groups were investigated; the number
2 of treatments is limited due to the fact that prepared bacteria might dye with the time.

3 An augmenting effect of hydrogen peroxide is confirmative to recent studies using a
4 different photosensitizer. Hydrogen peroxide when combined with toluidine blue increased
5 bactericidal activity of aPDT by using an LED emitting in the red spectrum on a 6-species
6 mixtures of planktonic periodontopathogens [18].

7 Applying hydrogen peroxide potentiated the aPDT effect also on bacteria within
8 biofilms. The reduction of bacterial counts in biofilms formed on polystyrene plates was
9 dependent first on hydrogen peroxide concentration used before illumination and second on
10 time of illumination. The additional reduction following application of 3% hydrogen peroxide
11 was about 4 log₁₀, which is similar to a recent study using toluidine blue or methylene blue
12 combined with the respective illumination on 24 h-old biofilms [18]. Hydrogen peroxide may
13 act on biofilm matrix, applying nanoparticles with catalytic activity for generation of hydrogen
14 peroxide on *S. mutans* biofilm simultaneously degraded extracellular matrix and killed bacteria
15 within biofilm [35]. However, a clear effect of hydrogen peroxide on biofilm quantity and
16 metabolic activity of biofilms was not demonstrated in our study.

17 In an ex-vivo model simulating an endodontic infection, pretreatment of hydrogen
18 peroxide before applying methylene blue as photosensitizer and the respective illumination
19 increased the killing activity of aPDT by 1.5 log₁₀ [17]. In clinical studies, hydrogen peroxide
20 was used as an irrigate after applying phenothiazine chloride as photosensitizer, in initial peri-
21 implantitis this therapy was successful as using local antibiotics [36].

22 In vitro-results showed an inferiority in bactericidal activity of aPDT with riboflavin as
23 photosensitizer when comparing with aPDT using toluidine blue with a light emitting in the red
24 spectrum [16, 37]. To the authors best knowledge, no clinical study has yet evaluated the effect
25 of photodynamic therapy by using riboflavin as a photosensitizer. The “pocket model” used in
26 our study allows the use of mechanical instrumentation and also aPDT, thus mimicking the in-
27 vivo situation. The instrumentation only reduced cfu counts by about 60%. The finding that the
28 combination of mechanical instrumentation and aPDT used after the application of 3%

hydrogen peroxide killed all bacteria within the biofilm provides “proof of concept” for the potential antimicrobial effect of this approach and warrants clinical testing, e.g. in treatment of residual pockets in supportive periodontal therapy.

5. Conclusion

The in-vitro study suggests that aPDT using riboflavin and blue LED light applied after mechanical removal of biofilm and adjunctive 3% hydrogen peroxide solution should be evaluated further. It appears to represent a novel alternative for antimicrobial periodontal therapy.

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Figures legends

Fig. 1

Viable bacterial counts of selected bacteria after applying hydrogen peroxide and antimicrobial photodynamic therapy

Viable bacterial counts were determined as colony forming units (cfu) after applying 0.1% riboflavin as photosensitizer for 1 min and light exposure using an LED lamp emitting in the blue spectrum with a power peak at 460 nm for 30 s (P30) or 60 s (P60) and pretreatment with 0.25% (0.25H) or 3% (3H) hydrogen peroxide for 1 min.

A: *Streptococcus gordonii* ATCC 10558; B: *Actinomyces naeslundii* ATCC 12104; C: *Fusobacterium nucleatum* ATCC 25586; D: *Parvimonas micra* ATCC 33270; E: *Campylobacter rectus* ATCC 33238; F: *Porphyromonas gingivalis* ATCC 33277; G: *Prevotella intermedia* ATCC 25611; H: *Tannerella forsythia* ATCC 43037

Fig. 2

Biofilms on well-plates after applying hydrogen peroxide and antimicrobial photodynamic therapy

Viable bacterial counts (colony forming units (cfu)) (A), metabolic activity (B) and biofilm quantity (C) were assessed after applying 0.1% riboflavin as photosensitizer for 1 min and light exposure using an LED lamp emitting in the blue spectrum with a power peak at 460 nm for 30 s (P30) or 60 s (P60) and pretreatment with 0.25% (0.25H) or 3% hydrogen peroxide (3H) for 1 min.

** p< 0.01 vs. control

Fig. 3

Biofilms after applying instrumentation, hydrogen peroxide and antimicrobial photodynamic therapy in a periodontal pocket model

Viable bacterial counts (colony forming units (cfu)) were assessed after instrumentation (mechanical biofilm removal (M)), applying 0.1% riboflavin as photosensitizer for 1 min and light exposure using an LED lamp emitting in the blue spectrum with a power peak at 460 nm for 60 s (P60) and pretreatment with 0.25% (0.25H) or 3% (3H) hydrogen peroxide for 1 min. The biofilms were formed on dentin specimens and placed in an artificial periodontal pocket, where therapy was applied.

** p< 0.01 vs. control

1 Table 1

2 Series of experiments (killing of planktonic bacteria, activity on biofilm formed in 96-well-
3 plates, activity on biofilms placed in an artificial periodontal pocket) and included treatment
4 groups

#	Treatment groups	Planktonic bacteria	Biofilm in 96- well-plates	Biofilm in artificial periodontal pocket
1.	Control (0.9% w/v NaCl)	X	X	X
2.	PS + 30 s light	X	X	
3.	PS + 60 s light	X	X	X
4.	0.25% H ₂ O ₂ + PS + 30 s light	X	X	
5.	0.25% H ₂ O ₂ + PS + 60 s light	X	X	X
6.	3% H ₂ O ₂ + PS + 30 s light	X	X	
7.	3% H ₂ O ₂ + PS + 60 s light	X	X	X
8.	Mechanical biofilm removal			X
9.	Mechanical biofilm removal + 0.25% H ₂ O ₂ + PS + 60 s light			X
10.	Mechanical biofilm removal + 3% H ₂ O ₂ + PS + 60 s light			X

5 PS – photosensitizer (0.1% riboflavin); 30 s (60 s) light – 30 s (60 s) of applying light by
6 means of an LED lamp emitting in the blue spectrum

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